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ORIGINAL RESEARCH PAPER

Evaluation of inhibitory effect and apoptosis induction of Zyzyphus Jujube on tumor cell lines, an in vitro preliminary study

Fatemeh Vahedi · Mohsen Fathi Najafi · Kazem Bozari

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Abstract In the present study, water extract of dried fruit of Zyzyphus Jujube was tested for its possible anticancer effect and induction of apoptosis on human tumor cell lines, HEp-2, HeLa and Jurkat cell lines. The inhibitory effect of water extract of this fruit on cell proliferation was assessed by MTT colorimetric assay. The induction of apoptosis of this extract was analyzed by DNA fragmentation analysis. Zyzyphus Jujube extract showed inhibitory effects on mentioned cell lines. Jurkat leukemic line was found the most sensitive cells with IC50 of 0.1 μg mL^{-1}. Our study also showed a typical DNA laddering in this cell line. The present study showed cytotoxic activity of Zyzyphus Jujube on tumor cells. Although Zyzyphus Jujube has useful compounds for medical applications.

Keywords Apoptosis · Tumor cell line · Zyzyphus Jujube

Abbreviations

BSA	Bovine serum albumin
DMSO	Dimethyl sulphoxide
FBS	Fetal bovine serum
MTT	3-(4,5-dimethylthiazoyl)-2,5-diphenyltetrazolium bromide
PBS	Phosphate buffer saline

F. Vahedi (✉) · M. Fathi Najafi · K. Bozari
Biotechnology Department, Vaccine and Serum Research Institute, Mashhad, Iran
e-mail: vahedif@yahoo.com

Introduction

Use of plants as medicine is recorded at least to the Middle Paleolithic age some thousands years ago by fossil date (Sampson 2005). It is estimated by the World Health Organization that ~75–80% of the world's population uses plant medicines either in part or entirely (Centers for Disease Control and Prevention 1995). Growing numbers of consumers are turning to plant medicines for many reasons—low cost and seeking natural alternatives with fewer side effects such as toxicity are commonly cited (Bown 1995). Plant-derived compounds have been an important source of several clinically useful for the treatment or prevention of diseases, including cancers (Gonzales and Valerio 2006).

In recent years, the discovery of novel chemopreventive agents of natural origin has been targeted, with fruits and vegetables being of key interest. The beneficial effects of fruits and vegetables have been attributed among other things to the high content of bioactive compounds (Rafter 2002). Studies conducted in the last two decades have shown that bioactive compounds have important roles in the prevention of chronic diseases (Colic and Pavelic 2002; Huang and Pardee 1999).

Cancer has long ranked as the most common diseases causing death worldwide in the view of the general public and many health professionals. According to the World Health Organization, physicians currently diagnose 10 million new cases of

cancer each year. Statistical trends indicate that this number will double by 2020 (Mignogna et al. 2004). Although chemotherapy is the standard method of treatment for patients, it has not been fully effective. Therefore development of new agents is still important to reduce the rate of mortality. Plants have been demonstrated to be a source of clinically relevant anticancer compounds (Mans et al. 2000).

Common jujube (Zyzyphus Jujube) is a plant native to Asia and Southern Europe. The plant belongs to the Rhamnaceae family (Omidbaigi 2005). The mature fruit is red to purplish-black and wrinkled, looking like a small date (hence the name Chinese date, Red date). The fruit has a single hard seed, similar to an olive stone. The dried fruit is known as *Annab*, in Persian. It is used as a delicious fruit and an effective herbal remedy. The high content sugar of fruit, explains its very sweet taste.

Jujube is a valuable medicinal plant in Iranian traditional medicine and is mentioned as a laxative and blood purifier. Jujube grows in arid and semi-arid zones of Iran especially in South Khorasan province, Birjand. In China, it is used as a taste enhancer and it is recommended for treating fatigue, loss of appetite and diarrhea. It is believed the dried fruits of Zyzyphus Jujube are anodyne, anticancer, pectoral, refrigerant, sedative, stomachic, styptic and tonic and immune response enhancer (Bown 1995; Duke and Ayensu 1985; Him-Che 1985).

The seeds of Ziziphus jujuba are used as a traditional medicine. Different compounds have been isolated previously from this genus. Some of these compounds exhibit significant pharmacological activities (Duke and Ayensu 1985).

In the present study we investigated the cytotoxic activity of Zyzyphus Jujube, have not been studied previously. The possible mechanism of this fruit and possible induction of apoptosis in cell lines is described.

Materials and methods

Reagents

Ficoll-Hypaque and bovine serum albumin (BSA) were purchased from Gibco BRL (Grand Island, NY, USA). RPMI 1640, Ampicillin, 3-(4,5-dimethylthiazoyl)-2,5-diphenyltetrazolium bromide (MTT), fetal bovine serum (FBS) and other reagents were purchased from Sigma (St. Louis, MO, USA).

Preparation of water extract of Zyzyphus Jujube

The semi-dried fruits of Zyzyphus Jujube were washed and then their seed were separated from soft red parts and removed. The samples were dried in 50 °C and were grounded into powder in a mortar. Extract was obtained by maceration of the powder in distilled water with stirring at room temperature for 2 h. The obtained slurry extract was boiled for 2 h and then was centrifuged in 7,000*g* for 30 min to remove insoluble ingredients. Then supernatant was filtered by Whatman No. 2. A part of filtered supernatant was subjected to dialysis against phosphate-buffered saline (PBS) solution (pH = 7.2, 0.15 M) to remove soluble sugars. Both kinds of samples, dialyzed (D) and non-dialyzed (ND), were lyophilized and kept at 4 °C. Dried extracts were later dissolved in RPMI medium to obtain 20 mg mL^{-1} and mixed at 37 °C for 20 min. These solutions were passed through 0.22 μm filters to sterilize and were diluted with the medium and prepared at different concentrations.

Protein content was determined in two kind samples (D and ND) with mini-Bradford assay and BSA was used as standard (Bradford 1976). Carbohydrate level was quantized using the phenol–sulphuric acid assay and glucose and fructose were used as standard (Dubois et al. 1956).

Cell culture

HeLa (the human cervical carcinoma cell line), HEp-2 (human larynx carcinoma cell line) and Jurkat (T cell leukemia) cell lines from Iranian cell bank, Pasteur Ins., Iran, were used in this study. All the cell lines were kept in RPMI 1640 medium supplemented with 10% FBS serum in culture flasks at 37 °C in 5% humidified CO_2 incubator. The cells were fed until confluence (2×10^6) and expanded by trypsinization, for adherent cells, (HeLa and HEp-2) and subcultured at lower numbers in new culture flasks. Cell proliferation was determined by counting viable cells using Trypan blue exclusion as the basis of viability (Coligan 1990).

Lymphocyte isolation and culture

Lymphocytes of normal human, as control cells, from heparinized blood were isolated by Ficoll-gradient centrifugation, were washed three times in PBS solution and resuspended in RPMI 1640 medium supplemented with 2 mM L-glutamin, 100 U mL^{-1} Penicillin G, 100 μg mL^{-1} Streptomycin, and 10% FBS inactivated for 30 min at 56 °C. Lymphocytes were cultured for 24 h in 95% humidified air containing 5% CO_2 (37 °C) (Beyum 1968).

DNA fragmentation analysis

The isolation of fragmented DNA from cells was done by a method described, previously (Herrmann et al. 1994). In brief, cells (2×10^6) were treated with the Zyzyphus Jujube extract and then collected by centrifugation (2,000*g*, 10 min). The pellet was resuspended in 0.5 mL DNA lysis buffer (2% SDS, 10 mM EDTA, 10 mM Tris–HCl, pH = 8.5). The lysate was incubated with 0.1 mg mL^{-1} Proteinase K and then incubated for 4 h at 37 °C. The DNA was precipitated with 70% ethanol after addition of isopropanol. Then the suspension was centrifuged and DNA treated with 0.5 mg mL^{-1} Rnase A in 200 μL of 10 mM Tris–HCl (pH = 7.5) at 37 °C for overnight. The sample was analyzed by electrophoresis on agarose gel in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA), containing ethidium bromide and was run in TAE buffer. The gel was photographed under UV illumination.

Morphological changes of cells after treatment

After treating of cells with Zyzyphus Jujube extract, observation was done under inverted microscope. Any change in morphological appearance of cells, concluding shrinkage, detachment, and colony forming of cells, was considered in evaluation of cells.

Treatment of cultured cells and MTT colorimetric assay

A colorimetric assay using MTT was performed (Mosmann 1983). Briefly, Cells were seeded in 96-well microplates (5,000 cells per well in 200 μL) and routinely cultured in a humidified incubator for 24 h. After a 24 h pre-culture, the medium was aspirated off, and exchanged for that containing Zyzyphus Jujube extract at various concentrations ranging from 0.001 to 1,000 μg mL^{-1}. Then cells were re-incubated for 72 h. In this test, a control group (RPMI without extract) and blank groups (without cells) were also included. This assay was performed in triplicate. The medium with or without extract was then discarded, and 100 μL of MTT solution (0.5 mg mL^{-1} in RPMI) were added to every well. Cells were re-incubated for an additional 2 h. Then the culture media were discarded and 0.15 mL dimethyl sulphoxide (DMSO) was added. The plates were vibrated for 10 min to dissolve the formazan crystals. Then, the optical density of 96-well culture plates was measured using an enzyme-linked immunosorbent assay (ELISA) reader at 540 nm. The optical density of untreated control cells was taken as 100% of viability. The growth inhibitory rates were calculated according to the following formula:

$$\text{Growth inhibitory rate } (\%) = (1 - \text{absorbance of the treated wells})/(\text{absorbance of the control wells}) \times 100\%.$$

Statistical analysis

The effects of extracts were expressed by IC50 (50% inhibition of cell proliferation) values calculated from dose–response curves by computer program (Graph Pad Prism). Student's *t*-test was used for statistical analyses; *P* values <0.05 were considered to be significant.

Results

Effect of dialysis on protein and sugar contents

The yield of extraction was calculated about 15% (w/w). Assessment of protein and sugars showed no change in protein content but about 90% of sugars have been removed after dialysis (Fig. 1).

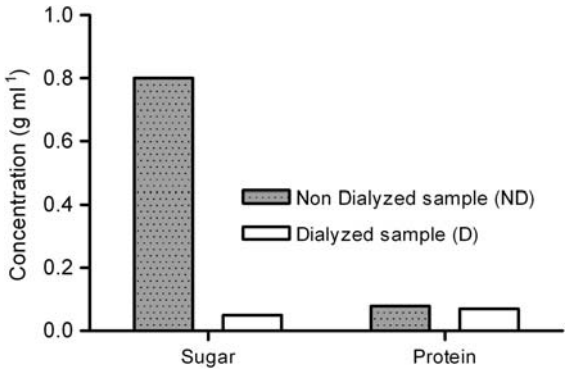

Fig. 1 The protein and carbohydrate contents of Zyzyphus Jujube extract were assessed by Bradford and Phenol sulphuric methods, respectively. About 90% of sugars have been removed after dialysis. The protein content of dialyzed sample (D) was found equal before dialyzing (ND)

Effect of the extracts on the growth and morphology of cultured cells

Shrinkage and detachment were observed in adherent cells, HeLa and HEp-2. These effects were increased by increasing in dose of extract. The number of colony forming cells was decreased in Jurkat cell line obviously. This inhibitory effect was found stronger with using dialyzed extract. Normal lymphocytes only suppressed in high dose (100 μg mL^{-1}). Most sensitive cell was found Jurkat cell line (Fig. 2).

Effect of the extracts on the cell proliferation, MTT assay

The inhibition of proliferation was tested by MTT assay following the treatment of different cell lines with the extracts. Under experimental conditions, Jurkat cell line exhibited decrease in growth in a dose-dependent manner (Fig. 3). According to 50% inhibition of cell proliferation (IC50), the order of sensitivity of the cell lines to this extract was Jurkat > HEp-2 > HeLa > normal lymphocyte cells. Obtained IC50 for Jurkat HEp-2 and HeLa cell lines were 0.1, 10 and 20 μg mL^{-1}, respectively. For normal lymphocyte cells, it was 100 μg mL^{-1}.

Effect of the extracts on DNA fragmentation

Incubation of Jurkat cells with extracts showed typical DNA ladder by electrophoresis after 24 h. We did not find the DNA ladder shape in other cells (Fig. 4).

Discussion

In this study, we analyzed the cytotoxic effect of water extract of Zyzyphus Jujube on the in vitro growth of human tumor cell lines, HeLA, HEp-2

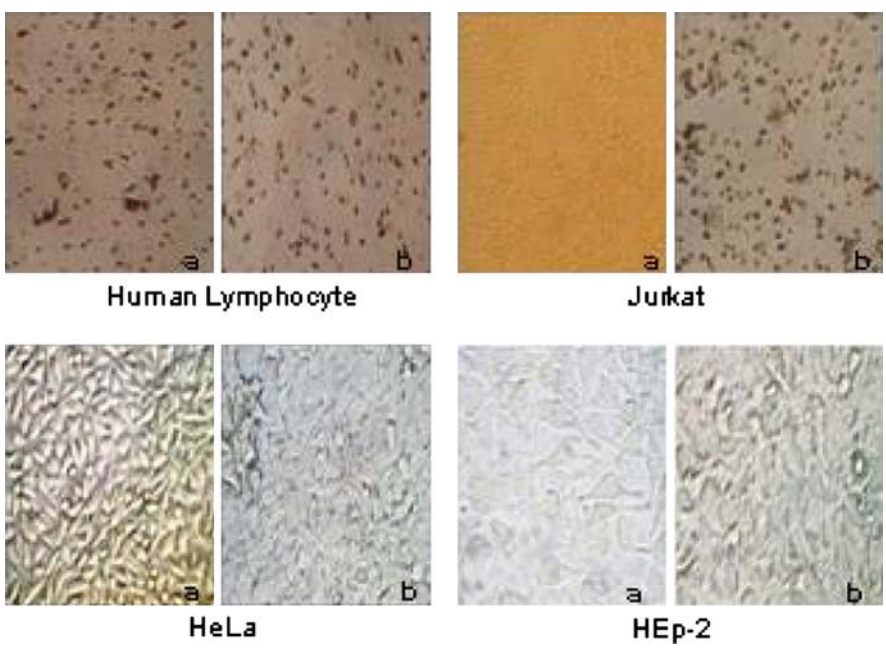

Fig. 2 Morphological changes of normal human lymphocyte, Jurkat, HeLa and HEp-2 cells after treating with Zyzyphus Jujube (50 μg mL^{-1}) for 24 h including shrinkage, detachment and non-colony forming when compared with untreated cells (a, untreated cells; b, treated cells)

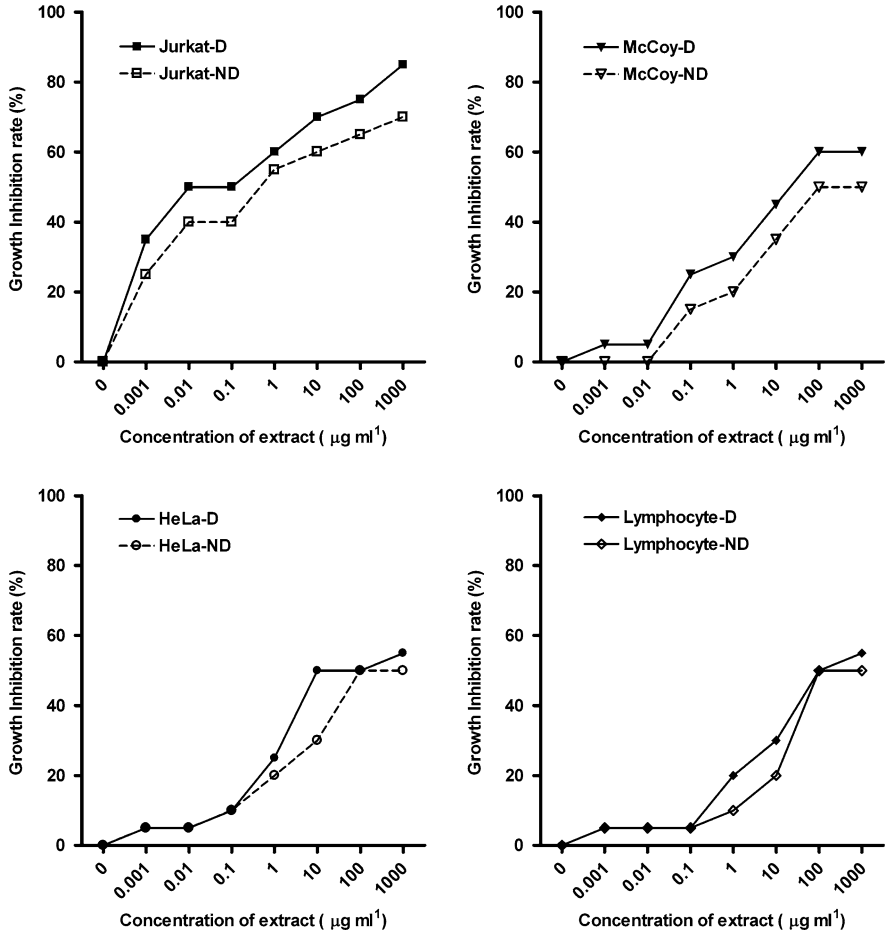

Fig. 3 Effects of Zyzyphus Jujube extracts on the growth of cells. Cells were seeded into 96-well plates. After incubation for 24 h, the cells were incubated with the culture medium containing various concentrations of Zyzyphus Jujube for 72 h. Each point represents the mean ± SD ($n = 3$); D, Dialyzed sample; ND, Non-dialyzed sample

and Jurkat cell lines, representing different tumor types.

Detachment of adherent cells, HeLA and HEp-2, was seen obviously and by MTT assay, it was confirmed that the number of viable cells has been decreased after treatment in all tumor cell lines. The most inhibitory activity of Zyzyphus Jujube was found on Jurkat leukemia cell line indicating the strong anti proliferative activity of the extract against this type of tumor cells. The results demonstrate the dose dependent cytotoxic effects of extract on cells. As our study showed, HeLa and HEp-2 cells, which originate from epitheloid cervix and human larynx carcinoma, respectively, were less affected by this extract.

The dialyzed extract showed higher activity against cells. It would be speculated that the some constituents like sugars in non-dialyzed extract have growth enhancement effect on the cells. The sugars levels in dialyzed extract were found very less than non-dialyzed extract (90%). Considering the sugar is as an important factor in growth of cells, it would be possible, the inhibitory effect has been covered, partially using non-dialyzed extract.

All the tumor cell lines responded to the extracts in a similar fashion, morphologically, perhaps indicating a common mechanism of action of the extract in all the cell lines used.

It has been shown that some anti neoplastic effects of the medicinal herbs are based on apoptosis induction (Cuendet et al. 2004; Dorrie et al. 2001; Huang and Pardee 1999; Malik et al. 2002). Apoptosis is a highly regulated process that occurs as part of differentiation, proliferation and growth of normal and malignant cells and plays a critical role in tumor initiation, progression, as well as in cancer therapy (Kim et al. 2002; Ogbourne et al. 2004).

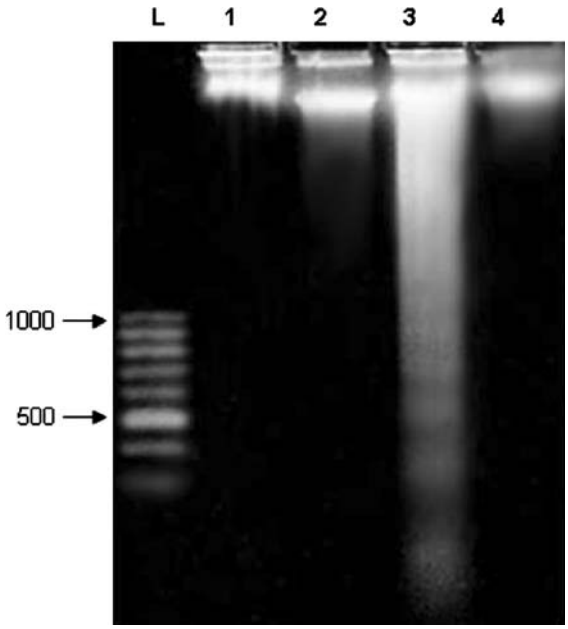

Fig. 4 Electrophoretic pattern of separated DNA from treated cells with Zyzyphus Jujube (50 μg mL^{-1}). DNA laddering typical for apoptotic cells is visible for treated Jurkat cells with the extract (L: Ladder, 1: HeLa, 2: HEp-2, 3: Jurkat, 4: Normal human lymphocyte)

Whether the anti proliferative activity observed for the Zyzyphus Jujube extract is due to the induction of apoptosis was examined in our study. Results of DNA fragmentation showed Zyzyphus Jujube has the ability to induce apoptosis in Jurkat cells (Ogbourne et al. 2004). Previously, the anti proliferative activity in gastric tumors in nude mice has been demonstrated for CKBM, a mixture of natural herbs containing Zyzyphus Jujube. The in vitro cytotoxicities of the triterpenoic acids derivates from Zyzyphus Jujube against K562, B16 (F-10), SK-MEL-2, PC-3, LOX-IMVI, and A549 tumor cell lines were investigated (Lee et al. 2003). We did not find DNA fragmentation in other two tumor cell lines after treatment with the extract. It may be related to contribution another mechanism in cell death.

In conclusion, our data showed that Zyzyphus Jujube has cytotoxic activity on different tumor cell lines. The capacity of this fruit to induce apoptosis in T cell leukemic cell lines candidate them for further studies in order to find the active components that are involved and the mechanism by which they induce apoptosis.

However, further studies are needed to examine the pharmacokinetics and the therapeutic action of Zyzyphus Jujube fruit on tumor cells.

References

Beyum A (1968) Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Lab Invest 21(Supp 97):77–89

Bown D (1995) Encyclopaedia of herbs and their uses. Dorling Kindersley, London

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Centers for Disease Control, Prevention (1995) Self-treatment with herbal and other plant-derived remedies-rural Mississippi. 1993. 204–207

Colic M, Pavelic K (2002) Molecular, cellular and medical aspects of the action of nutraceuticals and small molecules therapeutics: from chemoprevention to new drug development. Drugs Exp Clin Res 28(5):169–175

Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W (1990) Trypan blue exclusion test of cell viability. Current protocols on immunology. Wiley, New York

Cuendet M, Christov K, Lantvit DD et al (2004) Multiple myeloma regression mediated by bruceantin. Clin Cancer Res 10(3):1170–1179

Dorrie J, Sapala K, Zunino SJ (2001) Carnosol-induced apoptosis and downregulation of Bcl-2 in B-lineage leukemia cells. Cancer Lett 170(1):33–39

Dubois MG, Gilles KA, Hamilton JK, Rebers PA et al (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356

Duke JA, Ayensu ES (1985) Medicinal plants of china. Reference Publications, Institute of Chinese Medicine, S219–S224

Gonzales GF, Valerio LG (2006) Medicinal plants from Peru: a review of plants as potential agents against cancer. Anticancer Agents Med Chem 6(5):429–444

Herrmann M, Lorenz HM, Voll R et al (1994) A rapid and simple method for the isolation of apoptotic DNA fragments. Nucleic Acids Res 22(24):5506–5507

Him-Che Y (1985) Handbook of Chinese herbs and formulas. Institute of Chinese Medicine, S219–S224

Huang L, Pardee AB (1999) Beta-lapachone induces cell cycle arrest and apoptosis in human colon cancer cells. Mol Med 5(11):711–720

Kim R, Tanabe K, Inoue H, Toge T (2002) Mechanism(s) of antitumor action in protracted infusion of low dose 5-fluorouracil and cisplatin in gastric carcinoma. Int J Oncol 20(3):549–555

Lee SM, Min BS, Lee CG et al (2003) Cytotoxic triterpenoids from the fruits of Zizyphus jujuba. Planta Med 69(11):1051–1054

Malik A, Kuliev ZA, Akhmedov UA et al (2002) New oligomeric proanthocyanidine from Ziziphus jujuba. Chem Nat Compd 38(1):40–42

Mans DR, da Rocha AB, Schwartsmann G (2000) Anti-cancer drug discovery and development in Brazil: targeted plant collection as a rational strategy to acquire candidate anti-cancer compounds. Oncologist 5(3):185–198

Mignogna MD, Fedele S, Lo Russo L (2004) The World cancer report and the burden of oral cancer. Eur J Cancer Prev 13(2):139–142

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63

Ogbourne SM, Suhrbier A, Jones B et al (2004) Antitumor activity of 3-ingenyl angelate: plasma membrane and mitochondrial disruption and necrotic cell death. Cancer Res 64(8):2833–2839

Omidbaigi R, Daghighi S (2005) Effects of sucker age and transplanting time on the propagation of jujube tree. Paper presented at the III WOCMAP congress on medicinal and aromatic plants, Thailand

Rafter JJ (2002) Scientific basis of biomarkers and benefits of functional foods for reduction of disease risk: cancer. Br J Nutr 88(Suppl 2):S219–S224

Sampson W (2005) Studying herbal remedies. N Engl J Med 353(4):337–339